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Phenotype and immunoglobulin gene configuration of blood B cells from patients with multiple myeloma

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SUMMARY

We have studied the phenotype and the immunoglobulin gene configuration of blood B cells from 15 patients with stage III multiple myeloma (MM) at diagnosis. Highly purified B cells (>90% CD20 positive cells) were obtained after L-leucine methyl ester monocyte depletion and elimination of T cells by rosetting. The percentage of B cells with surface immunoglobulin (sIg) featuring the same light and heavy chain isotype as the serum monoclonal immunoglobulin was very low, except in one patient, in whom 25–30% of B cells displayed surface and cytoplasmic immunoglobulin (cIg) sharing idiotypic determinants with the serum monoclonal IgGκ. In all cases but one the percentage of circulating plasma cells accounted for less than 2% of the enriched B cell preparations. In one patient purified B cell population contained 30% of plasma cells and the immunoglobulin gene study revealed a rearranged J_H hybridizing fragment identical in bone marrow and blood B cell DNA samples. In the other 14 cases no rearranged fragment was detected although we used a technique allowing the detection of at least 2% clonal cells. The absence of clonal cells in the patient whose B cells contained a high percentage of cells featuring surface IgG molecules was confirmed on purified sIgG-positive cells. In addition CD20-positive cells from this patient did not contain γmRNA. Therefore the IgG molecules were clearly extrinsic. Although the existence of clonal B lymphocytes or of myeloma idiotype related B cells cannot be ruled out, they escape detection by sensitive genetic studies of immunoglobulin genes.

Keywords multiple myeloma immunoglobulin gene

INTRODUCTION

Several data suggest that various populations of circulating B cells are related to the clonal process in patients with multiple myeloma (MM). Studies with monospecific anti-immunoglobulin and/or idiotypic sera provided strong evidence that a sizeable percentage of blood B cells feature surface immunoglobulin (sIg) which are identical (or which share idiotypic determinants) with the serum monoclonal immunoglobulin (Seligmann, Preud'homme & Brouet, 1973; Mellstedt, Hammarström & Holm, 1974; Abdou & Abdou, 1975; Kubagawa *et al.*, 1979; Schedel *et al.*, 1980; Boccadoro *et al.*, 1981; van Camp, Reynaert & Broodtaerts, 1981; Bast *et al.*, 1982). Furthermore, pre-B cells have been implicated in the disease process in two different reports (Kubagawa *et al.*, 1979; Grogan *et al.*, 1987). Finally circulating plasma cell precursors may be induced to grow and differentiate in vitro in the presence of IL-6 and IL-3 (Bergui *et al.*, 1989).

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However, the presence of blood clonal B cells as assessed by the study of immunoglobulin gene rearrangements is controversial (Berenson *et al.*, 1987; Clofent *et al.*, 1989; Knauf *et al.*, 1989; Van Riet *et al.*, 1989). These studies did not mention detailed phenotypes of the cell populations studied, nor investigated rigorously T and monocyte depleted B cells. Here we looked for clonal rearrangements of immunoglobulin genes in highly enriched B cell population (>90% B cells) from 15 patients with MM. No evidence of circulating non-plasmacytic clonal B cells was obtained.

MATERIALS AND METHODS

Patients

Fifteen patients with high tumour mass MM, stage III of the Durie & Salmon (1977) classification, were studied before any treatment. Eight patients had IgGκ, two had IgA (κ and λ in one case each) and four had light chain (three κ, one λ) MM. One patient had non-secretory MM. There were no circulating

plasma cells identified by an automated differential count of leucocytes.

Cell preparation

Blood or bone marrow mononuclear cells were isolated by Ficoll density gradient centrifugation (Lymphoprep; Nyegaard, Oslo, Norway). Purified blood B cell preparations were obtained after depletion of monocytes by treatment of peripheral blood mononuclear cells (PBMC) with L-leucine methyl ester (Sigma, St Louis, MO) (Thiele, Kurasaka & Lipsky, 1983) and elimination of T cells by rosetting with AET-treated sheep erythrocytes. Bone marrow cell samples contained 5–20% malignant plasma cells by cytological examination.

In patient A a subset of B cells featuring membrane-associated IgG molecules was isolated by panning. Briefly, purified B cells were stained for 30 min at 4°C with a monoclonal antibody (MoAb) to human γ heavy chain and thereafter poured in to a plastic Petri dish previously coated with affinity purified goat Fab'_2 fragments of IgG to mouse immunoglobulin (Biosys, Compiegne, France). After incubation for 1 h at 4°C, non-adherent B cells (surface IgG-negative B cells) were discarded and adherent B cells were collected after several flushes of cold phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS).

In another set of experiments, blood B cells from the same patient A were depleted of contaminating plasma cells by complement dependent cytotoxicity using the PCA1 MoAb. CD20-positive B cells were thereafter purified using an Ortho cytofluorograph as described previously (Grillot-Courvalin, Labaume & Brouet, 1986).

Immunofluorescence analysis

Direct or indirect immunofluorescence analysis of sIg or cytoplasmic immunoglobulin (cIg) were performed as reported elsewhere (Preud'homme & Labaume, 1975). Fluorescein-conjugated Fab'_2 fragments of goat IgG to human immunoglobulin were purchased from Nordic (Tilburg, The Netherlands). Rabbit Fab'_2 fragments of IgG monospecific for human immunoglobulin light and heavy chains conjugated to fluorescein or rhodamine were prepared in our laboratory (Preud'homme & Labaume, 1975). Staining with MoAb was performed using a second layer of fluorescein-conjugated Fab'_2 fragments of rabbit IgG to mouse immunoglobulin extensively absorbed on human IgG.

An anti-idiotypic serum was obtained by immunization of rabbits with the Fab'_2 fragments of monoclonal IgGκ purified from the serum of patient A by immunoabsorption on protein A Sepharose beads. The immune serum was absorbed extensively on normal human IgG, IgM and κ chains coupled to Sepharose 4B and used for intracytoplasmic staining with a second layer of fluorescein-conjugated Fab'_2 fragments of goat IgG to rabbit IgG.

Analysis of immunoglobulin genes

High molecular weight DNA from purified blood B lymphocytes, T lymphocytes and bone marrow mononuclear cells was extracted (Davis, Dibner & Battey, 1986). Digestion of 20 μg of DNA samples was carried out with three different restriction enzymes (*Bam*HI, *Eco*RI, *Hin*dIII), and electrophoresed on agarose gel, followed by Southern blot transfer to nylon membrane (Hybond N, Amersham, France). The sensitivity of the Southern method we used was evaluated by mixing experiments: DNA was extracted from a mixture of normal PBMC and an increasing percentage of malignant cells from a patient with plasma cell leukaemia. Two per cent of clonal cells were detected (not shown).

For Northern analysis, 10 μg of total mRNA were isolated by the isothiocyanate guanidinium method (Chirgwin *et al.*, 1979). RNA samples from patient A cells or control IgGκ-producing cells from the BL42 line or from a patient with chronic lymphocytic leukaemia (CLL), were electrophoresed on 1% agarose formaldehyde gel and then blotted to nitrocellulose CE filter (Amersham).

The configuration of HH region was evaluated by hybridizing a 2·6 kb *Sau*3A joining region (JH fragment) to genomic DNA digested with *Bam*HI, *Eco*RI or *Hin*dIII (Tsapis *et al.*, 1989). γ mRNA was detected with a 600-bp cDNA probe containing the second and third constant domain of the γ3 heavy chain gene (Alexander *et al.*, 1982). Probes were radiolabelled with ^{32}P-dCTP using the random primer method (Feinberg & Vogelstein, 1984).

RESULTS

Characterization of B cell preparations

Enriched B cell preparations from 15 patients with stage III MM were obtained by depletion of monocytes and T lymphocytes. They contained 80–90% sIg and/or CD20 positive cells, i.e. an 8–20-fold enrichment over PBMC preparations. Most remaining cells were monocytes stained with the MoAb CD11b.

In most cases (13 out of 15 patients) B cells carried polyclonal μ and/or δ sIg; less than 2% cells expressed membrane IgG or IgA. There was no obvious dysbalance of light chain expression among sIg-positive B cells. Typical plasma cells, sometimes with dystrophic features were identified by cytoplasmic immunoglobulin staining or membrane expression of the PCA1 antigen. They accounted for less than 2% cells, except in patient B with IgGκ MM, whose enriched B cell preparation contained a high percentage (30%) of malignant plasma cells.

In patient A with IgGκ MM, a high percentage (25%) of small B cells featured surface and cytoplasmic IgGκ molecules. These cells were CD20-positive, PCA1-negative. Similar results were obtained on fresh cells as well as after 24 h of culture in RPMI FCS medium. A rabbit anti-idiotypic serum raised against the purified IgGκ myeloma protein stained 25–30% small B cells by cytoplasmic staining as well as the circulating or bone marrow plasma cells. In contrast, when B cells from this patient were stimulated by infection with the Epstein–Barr Virus (EBV) and studied at day 10, less than 1/1000 cells were stained, i.e. a percentage similar to that observed in five preparations of pokeweed-stimulated PBMC from different individuals. Surface IgG-positive B cells from this patient were purified by a panning procedure and studied for immunoglobulin gene configuration. In addition, CD20-positive B cells were sorted from the B cell-enriched preparation after depletion of PCA1-positive cells. This B cell population which contained 30% of small B cells featured by surface and cytoplasmic IgGκ molecules and no detectable plasma cells, was studied for the presence of γ mRNA. The phenotype of the various cell populations is shown in Table 1.

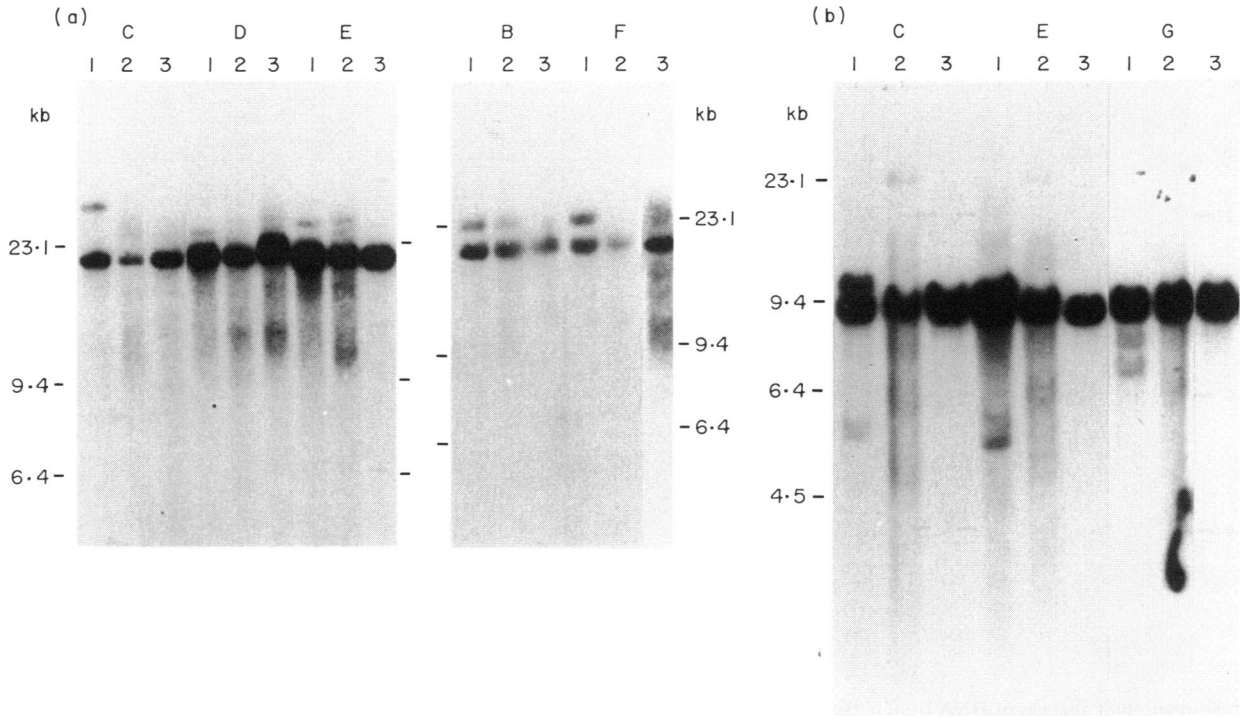

Fig. 1. Southern blot hybridization from representative patients with MM. (a) DNA, extracted from patients B–F was digested with *Eco*RI restriction enzyme; (b) DNA extracted from patients C, E, G was digested with *Hin*dIII restriction enzyme. Blots were hybridized with a J_H probe: (1) bone marrow mononuclear cells; (2) purified blood B lymphocytes; (3) T lymphocytes. A rearranged DNA fragment was detected in all bone marrow samples. Except in patient B, no rearranged band was detectable in other blood B cells DNA preparations.

Table 1. Blood B cell populations purified from patient A.

	Purified blood B cells (%)	sIgG$^+$ blood B cells (%)	CD20$^+$ PCA$_1^-$ blood B cells (%)
CD20	90	80	>99
PCA$_1$	2	<2	<0.1
IgGκ	25	80	30
Idiotype positive	25	ND	30

Blood B cells were obtained after L-leucine methyl ester monocytes depletion and T rosetting; sIgG$^+$ blood B cells were purified by a panning procedure; Purified blood B cells were depleted of contaminating plasma cells by complement-dependent cytotoxicity using PCA$_1$ MoAb. CD20$^+$ B cells were thereafter purified by a cell sorter procedure.

ND, not done; sIgG, surface IgG.

Genotypic studies

DNA extracted from purified blood B cells, bone marrow mononuclear cells and T lymphocytes were digested with *Bam*HI, *Eco*RI (Fig. 1a) or *Hin*dIII (Fig. 1b) restriction enzymes and hybridized with a J_H probe. Representative experiments are shown in Fig. 1. With one exception (patient B) no rearranged band was identified in all B cell DNA samples studied. As expected, clonal rearrangements were observed in all bone marrow DNA samples. Purified blood B cells from patient B which contained 30% plasma cells and bone marrow cells from the same patient exhibited identical rearranged J_H hybridizing bands (Fig. 1a).

No rearranged fragment was detected with JH and Cγ probes in DNA samples from patient A (Fig. 2) although the purified B cell population contained around 25% B cells with surface and cytoplasmic IgGκ molecules. Likewise, B cells with IgG-associated molecules isolated by panning with anti-γ heavy chain MoAb (more than 80% positive cells) displayed germline hybridizing fragments.

Northern blot analysis of patient A PBMC RNA showed the presence of a 1·8 kb secreted form of γ mRNA without detectable membrane γ mRNA. This mRNA most likely corresponded to the presence of circulating plasma cells; accordingly, no γ mRNA was present in CD20-positive, PCA1-depleted blood B cells (Fig. 3).

DISCUSSION

The presence of blood B cells belonging to the malignant myeloma clone is controversial. An increase in circulating B lymphocytes featured by sIg having the same isotype of light and heavy chains as serum monoclonal immunoglobulin has been observed in several myeloma patients (Seligmann *et al.*, 1973; Mellstedt *et al.*, 1974; Abdou & Abdou, 1975). In addition, the demonstration that serum monoclonal immunoglobulin and B cells sIg shared idiotypic determinants provided evidence that the cells belonged to the malignant clone (Kubagawa *et al.*, 1979; Schedel *et al.*, 1980; Boccadoro *et al.*, 1981; Bast *et al.*, 1982). However binding of the serum monoclonal immunoglobulin to PBMC receptors for immunoglobulin was not ruled

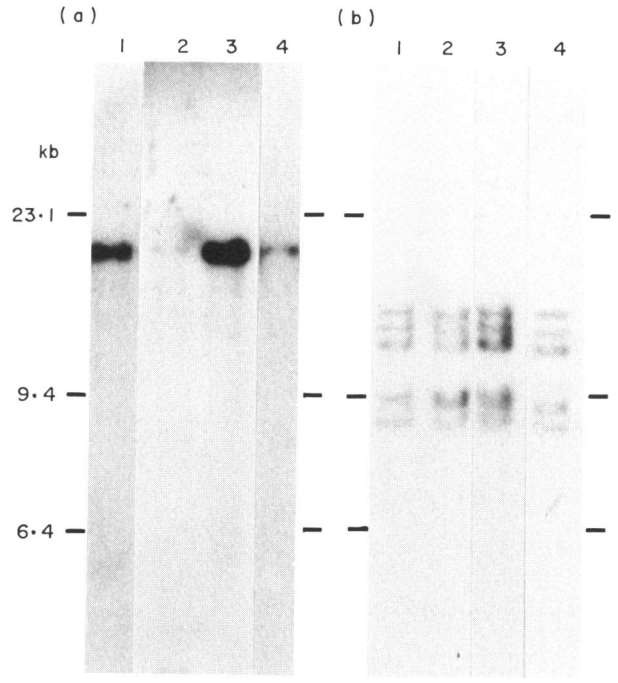

Fig. 2. Southern blot analysis of DNA from patient A cells. DNA was digested with *Bam*HI restriction enzyme and the filter was sequentially hybridized using J_H (a) and Cγ (b) probes. (1) sIgG-negative B cells (see Materials and Methods); (2) sIgG-positive B cells; (3) T cells; (4) enriched blood B cells. No rearranged fragment was detectable in DNA samples from patient A.

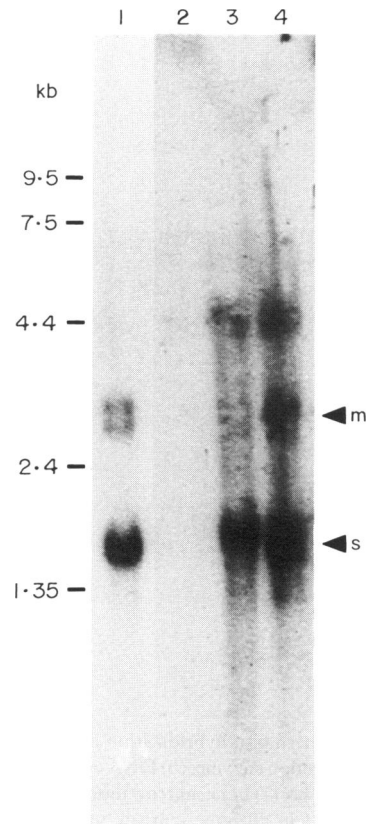

Fig. 3. Northern blot analysis of total RNA from patient A blood B cells. Blots were hybridized with a human Cγ probe. (1) RNA of cells from the BL42 IgG κ cell line; (2) RNA of CD20-positive, PCA1-depleted B cells; (3) RNA of patient A enriched B cells; (4) RNA of IgGκ CLL B cells. Arrows indicate secreted (s) and membrane (m) γm RNA forms. RNA for secreted γ heavy chain was detected in patient A enriched B cell preparation and not in the CD20-positive, PCA1-depleted preparation.

out in all studies. More recent studies aimed to detect clonal rearrangements of immunoglobulin genes in blood B cells yielded conflicting results (Berenson *et al.*, 1987; Clofent *et al.*, 1989; Knauf *et al.*, 1989; van Riet *et al.*, 1989). In order to solve some of these controversies, we studied highly purified blood B cells preparations (80–90% $CD20^+$ cells) from 15 patients with MM for both membrane and cytoplasmic immunoglobulin expression and immunoglobulin gene configuration. In 14 out of 15 patients, Southern blot analysis using sensitive procedures (samples of 20 μg DNA, several days of film exposure) did not detect any rearrangement in heavy chain genes using a J_H probe (Fig. 1) or light chain genes (not shown). In the patient B, there was a clonal rearrangement identical in blood and bone marrow samples; however, immunologic phenotype performed on the B cell enriched preparation disclosed a fair percentage of plasma cells which accounted for less than 1% blood cells. Therefore, in accordance with another study (Clofent *et al.*, 1989), we found no evidence for circulating clonal B lymphocytes in patients with MM. These results are at variance with three other studies which detected identical clonal rearrangements in both blood and bone marrow cells from 20% (Knauf *et al.*, 1989; van Riet *et al.*, 1989) to 80% (Berenson *et al.*, 1987) of cases. As there was no phenotypic study of the same preparation in these reports, the clonal population cannot be unequivocally attributed to B lymphocytes and not to circulating malignant plasma cells. Indeed, recent phenotypic studies emphasized the frequent invasion of blood by malignant plasma cells in progressive MM (Greipp, 1989).

In most patients studied here, enriched B cell preparations contained rare B cells with sIg or cIg having light and heavy chains identical to that of the serum monoclonal immunoglobulin. Therefore the study of patient A's B cells was of special interest since they included a sizeable (25%) percentage of cells with membrane and cytoplasmic associated IgG κ molecules which were idiotypically related to the monoclonal serum IgG κ. Although the phenotype of the cells was unchanged after *in vitro* culture, several experiments demonstrated that the IgG molecules were extrinsic: (i) there was no clonal J_H rearrangement among IgG enriched (80%) B cells; (ii) purified CD20 positive PCA1 depleted blood B cells displayed no γ mRNA; and (iii) no idiotypic cells were identified among EBV-induced IgG or IgM cIg cells after 10 days of *in vitro* culture. These data therefore indicate that the presence among B cells of myeloma patients of a large excess of cells with monotypic sIg as judged by immunologic analysis is likely to be artefactual.

Our study failed to confirm the presence of clonal, non-plasmacytic cells in blood from patients with MM, even using sensitive genetic techniques. However the presence of a very low number of clonal B cells or of myeloma idiotype-related polyclonal B cells (Kubagawa *et al.*, 1979) could not be excluded. The elucidation of the significance of such population must await their purification and long-term culture.

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